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(71) Applicant *(for all designated States except US)*: RESEARCH CORPORATION TECHNOLOGIES, INC. [US/US]; Suite 600, 101 North Wilmot, Tucson, AZ 84711-3335 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LAZAROVITS, Andrew, I. [CA/CA]; 63 Grasmere Crescent, London, Ontario N6G 4N7 (CA). POPPEMA, Sibrandes [CA/CA]; 12408 39th Avenue, Edmonton, Alberta T6G 0P3 (CA).

(74) Agent: VIKSNINS, Ann, S.; Schwegman, Lundberg, Woessner & Kluth, P.O. Box 2938, Minneapolis, MN 55402 (US).

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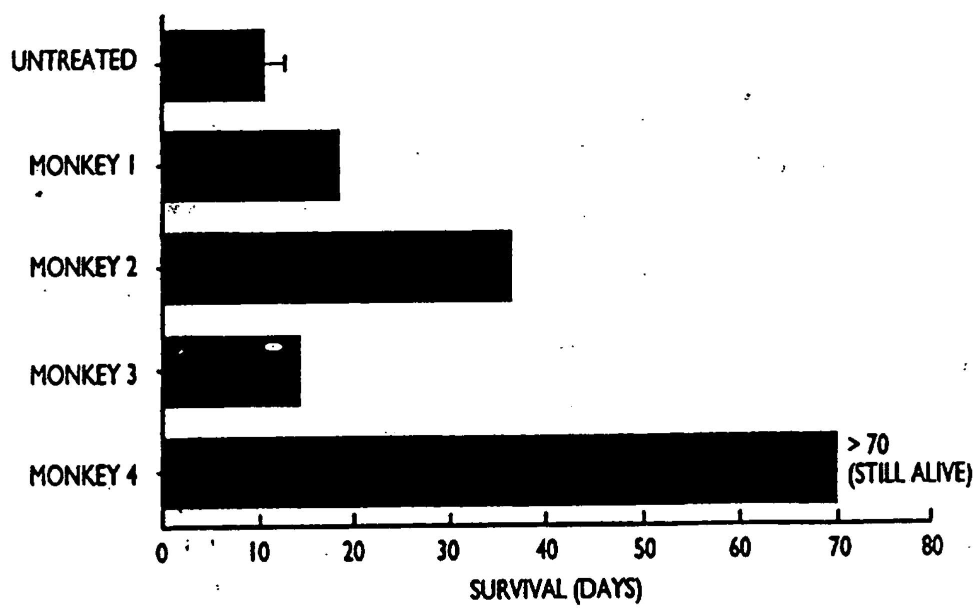

(57) Abstract

Comp[illegible]ylation of phospholipase C-γ1, for the prevention or reversal of transplant rejection, as well as therapy for autoimmune diseases.

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CD45RB binding compounds for the prevention of transplant rejection

Related application

This application is related to U.S. Serial No. 08/423,843 filed April 18, 1995, which in turn is a continuation-in-part application of U.S. Serial No. 08/071,009 filed June 2, 1993, both of which are herein incorporated by reference.

Field of the invention

This application relates to novel compounds and novel pharmaceutical compositions which are useful in modulating the immune response. The compounds comprise antagonists and agonists, especially to CD45RB antigens.

Background of the invention

Organ, cell and tissue transplant rejection and the various autoimmune diseases are thought to be primarily the result of a T-cell mediated immune response. This T-cell mediated immune response is initially triggered by helper T-cells which are capable of recognizing specific antigens. These helper T-cells may be memory cells left over from a previous immune response or naive cells which are released by the thymus and may have any of an extremely wide variety of antigen receptors. When one of these helper T-cells recognizes an antigen present on the surface of an antigen presenting cell (APC) or a macrophage in the form of an antigen-MHC complex, the helper T-cell is stimulated by signals emanating from the antigen-specific T-cell receptor, co-receptors, and IL-1 secreted by the APC or macrophage, to produce IL-2. The helper T-cells then proliferate. Proliferation results in a large population of T-cells which are clonally selected to recognize a particular antigen. T-cell activation may also stimulate B-cell

activation and nonspecific macrophage responses. Some of these proliferating cells differentiate into cytotoxic T-cells which destroy cells having the selected antigen. After the antigen is no longer present, the mature clonally selected cells will remain as memory helper and memory cytotoxic T-cells, which will circulate in the body and recognize the antigen should it show up again. If the antigen triggering this response is not a foreign antigen, but a self antigen, the result is autoimmune disease; if the antigen is an antigen from a transplanted organ, the result is graft rejection. Consequently, it is desirable to be able to regulate this T-cell mediated immune response.

CD45 antigen (CD45) is expressed on most leukocytes. Indeed, it was previously thought that a common CD45 antigen was present on all leukocytes, for which reason the receptor was originally known as the Leukocyte Common Antigen (LCA). Monoclonal antibodies to CD45 were proposed as a means of effectively eliminating all leukocytes where desirable, for example, purging an organ to be transplanted of passenger leukocytes prior to transplantation using nonspecific CD45 monoclonal antibody. See, *e.g.*, WO 91/05568.

It has recently been shown that different isoforms of CD45 are generated by alternate splicing of a single primary transcript of the CD45 gene. These CD45 isoforms include CD45RA, CD45RB, and CD45RO. CD45RA contains the expression products of exons 4, 5, and 6 (sometimes referred to as A, B, and C) of the CD45 gene; CD45RB contains the expression products of exons 4 and 5, but not 6; CD45RO does not contain the expression products of any of the three exons 4, 5, or 6. See Hall et al, "Complete Exon-Intron Organization of the Human Leukocyte Common Antigen (CD45) Gene," J. IMMUNOLOGY, Vol. 141, pp. 2781-2787 (1988), herein incorporated by reference and Streuli et al, "Characterization of CD45 and CD45R Monoclonal Antibodies Using Transfected Mouse Cell Lines that Express Individual Human Leukocyte Common Antigens," J. IMMUN., Vol. 141,

pp. 3910-3914, No. 11 (1988), herein incorporated by reference. The significance of this variable expression, however, has been unclear.

S. Poppema, et al., J. IMMUNOLOGY (July 1991) 147: 218, incorporated herein by reference, have previously described the monoclonal antibody MT3. This publication, however, does not disclose a detailed method for making this antibody, nor does it disclose any pharmaceutical use for this antibody and, therefore, necessarily fails to disclose the T-cell population recognized by this antibody. A. Lazarovits, et al., Transplantation (October 1992) 54: 724, incorporated herein by reference, has characterized the *in vitro* effect of this antibody. Lazarovits, for the first time, showed that MT3 mAb inhibits proliferation of and generation of T-cells by interfering with CD45RB (a leukocyte surface molecule).

Therefore, a need exists to effectively and selectively suppress or otherwise modulate the immune response in humans, particularly transplant recipients or those afflicted with autoimmune diseases.

Summary of the Invention

In a first embodiment, therefore, the present invention relates to CD45RB suppressors and pharmaceutical compositions containing the same which modulate the immune response in animals including humans by binding specifically to CD45RB, especially monoclonal antibodies to CD45RB and fragments of the monoclonal antibodies having such properties.

This invention is based on the discovery that different types of T-cells may predominantly express one or another of these CD45 isoforms of CD45 expression. Naive helper T-cells and memory T-cells express predominately CD45RA and CD45RO respectively. CD45RB expression is more variable, but it appears to be expressed on naive helper T-cells and especially

on naive cells which have been stimulated to proliferate. CD45RB expression is down-regulated as the cells become activated. It has now been shown that compounds which suppress CD45RB expression but not CD45RO expression are capable of selectively inhibiting the T-cell mediated immune response without destroying the pool of memory T-cells. CD45RB is of particular interest because if CD45RB function is suppressed, the proliferating cells will fail to mature, and as a result the antigen specific clone they comprise will be deleted. Consequently, CD45RB suppressors have a great advantage over current immunosuppressants in that (i) they act on a particular T-cell population rather than having an overall immunosuppressive effect, thereby avoiding the risk of side effects associated with over-suppression of the immune system; and (ii) they are capable of conferring long term tolerance to a particular antigen when they are administered contemporaneously with exposure to antigen, *e.g.*, just before and after an organ transplant or during an acute phase of an autoimmune disease.

In addition to MT3 mAb, Lazarovits reports that monoclonal antibodies to CD45RB, such as an antibody produced by the cell line HB220 which is publicly available from the ATCC in Rockville, MD (now designated anti-CD45RB mAb (MB23G2)), bind to CD45RB and are effective agents in inhibiting immune function *in vitro* and *in vivo* (see US Serial No. 08/071,009 filed June 2, 1993 herein incorporated by reference).

The inventors have now found that 6G3 monoclonal antibody binds to CD45RB. This is a murine IgG1 directed against human CD45RB. It cross-reacts with monkey CD45RB. The inventors have also now found that the MB23G2, 6G3, and MT3 monoclonal antibodies bind to neuraminidase sensitive epitopes on leukocytes including T-cells and that at least MB23G2 and 6G3 increase the tyrosine phosphorylation of phospholipase C-γ1. It is of interest to note that HB223 (now designated MB4B4), an analogous anti-CD45RB antibody to those of the invention, is found not to bind to neuraminidase sensitive epitopes. It is also observed that MB4B4 mAB

binds to a neuraminidase insensitive epitope and does not alter the tyrosine phosphorylation of phospholipase C-γ1. MB4B4 was also ineffective at preventing renal allograft rejection in mice.

Given these antibodies it is of course possible to alter or produce recombinant antibodies which may then be cloned and expressed by conventional techniques, *e.g.*, as described in EP 0 449 769 incorporated herein by reference. The terms antibody, antibody fragments, fusion compounds, chimeric compounds and derivatives thereof are collectively termed compounds or suppressors throughout the specification.

In a second embodiment, the present invention provides suppressor compounds and pharmaceutical compositions capable of modulating the immune response by competing with CD45RB for its binding site *in vivo*.

In yet another embodiment, the present invention relates to methods of *in vivo* immunosuppression in humans and other animal species. The methods include pretreatment *in vivo* therapies to prevent rejection of transplanted cells, tissues and organs and post-transplant *in vivo* therapies to reverse an immune response. The methods also include *in vivo* treatment of autoimmune diseases.

Brief Description of the Drawings

Figure 1 shows prolonged survival of MB23G2 - treated animals compared to an untreated group;

Figure 2 shows the serum creatine levels from each group at the time of sacrifice.

Figures 3A, 3B, and 3C shows that MB23G2 induces depletion of circulating lymphocytes and binds to the remaining T and B lymphocytes.

Figure 4 shows the survival of Cynomolgus monkeys treated with anti-CD45RB mAb 6G3, after receipt of allografts.

Detailed Description of the Invention

The suppressors of the invention are preferably produced as described in the following examples, or by equivalent means as would be apparent to one skilled in the art.

It will be understood by those skilled in the art that the hybridomas herein referred to may be subject to genetic mutation or other changes while still retaining the ability to produce monoclonal antibody of the same desired specificity. The present invention therefore encompasses mutants, other derivatives and descendants of the hybridomas.

It will be further understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other derivative antibodies, humanized or chimeric molecules or antibody fragments which retain the specificity of the original monoclonal antibody. Such techniques may involve combining DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of the monoclonal antibody with DNA coding the constant regions, or constant regions plus framework regions, of a different immunoglobulin, for example, to convert a mouse-derived monoclonal antibody into one having largely human immunoglobulin characteristics (see EP 184187A, GB 2188638A, herein incorporated by reference).

EP-A-0 120 694 (Boss et al/Celltech, herein incorporated by reference) describes the cloning and expression of chimeric antibodies. In these derivatives the variable domains from one immunoglobulin are fused to constant domains from another immunoglobulin. Usually, the variable domains are derived from an immunoglobulin gene from one species, for example a mouse or a rat, and the constant domains are derived from an immunoglobulin gene from a different species, perhaps a human. This technology is now very well known in the art. A later European Patent Application, EP-A-0 125 023 (Cabilly/Genetech, herein incorporated by reference), also U.S. Patent No. 4,816,567, describes much the same subject as the Boss patent

application, but describes production of other variations of immunoglobulin-type molecules using recombinant DNA technology.

Another possibility is to attach just the variable region of the monoclonal antibody to another non-immunoglobulin molecule, to produce a derivative chimeric molecule (see WO 86/01533, Neuberger and Rabbits/Celltech, herein incorporated by reference). A further possibility would be to produce a chimeric immunoglobulin having different specificities in its different variable regions, *e.g.*, the monoclonal antibodies of the present invention (see EP 68763a). Yet another possibility would be to produce a mutation in the DNA encoding the monoclonal antibody, so as to alter certain of its characteristics without changing its essential specificity. This can be done by site-directed mutagenesis or other techniques known in the art.

The Winter patent application EP-A-0 239 400 (herein incorporated by reference) describes how it is possible to make an altered, derivative, antibody by replacing the complementarity determining regions (CDRs) of a variable region of an immunoglobulin with the CDRs from an immunoglobulin of different specificity, using recombinant DNA techniques -- so called "CDR-grafting". This enables altering the antigen-binding specificity of an antibody. (In the present case it might be the CDRs of MT3, 6G3, MB23G2, an antibody with the same binding specificity as these anti-CD45RB antibodies, or an antibody which is cross-reactive with MT3, 6G3, or MB23G2 which are transferred to another antibody.) Thus, CDR grafting enables "humanization" of antibodies. A "humanized" antibody containing the CDRs of a rodent antibody specific for an antigen of interest might well be less likely to be recognized as foreign by the immune system of a human. It follows that a "humanized" antibody with the same binding specificity as, *e.g.*, MT3 or 6G3, or an antibody that cross-reacts with either (see later), might well be of particular use in human therapy and/or diagnostic methods. The use of

antibodies according to the present invention are discussed later in the text. All such uses of the monoclonal antibody are encompassed by the present invention.

As discussed, the state of the art is such that the person skilled in the art well knows how to manipulate and alter any given antibody or gene(s) encoding for the same to generate a derivative to suit his or her particular needs.

The provision of an antibody such as MT3 or 6G3 allows persons skilled in the art to obtain binding partners, *e.g.*, antigens/epitopes or antibody/paratopes which bind to it. Therefore, the present invention also provides binding partners, *e.g.*, antigens and/or antibodies which bind with an antibody or derivatives thereof as hereby provided, such as MT3 and 6G3.

The binding partners obtained by use of the MT3 mAb and 6G3 mAb may also be used to produce additional ligands, *e.g.*, antibodies other than MT3 or 6G3 (or molecules having antibody-like binding function, *e.g.*, fragments, derivatives and synthetic analogues of antibodies such as single-chain antigen-binding molecules). Therefore, also provided are ligands, *e.g.*, mAbs which are able to bind with a binding partner which is able to bind with the MT3 mAb and 6G3 mAb. Such ligands ("cross-reactive ligands") , *e.g.*, mAbs may recognize the same epitope as recognized by MT3 mAb and 6G3 mAb on said binding partner.

The present invention also provides derivatives, functional equivalents (*e.g.*, a molecule having an antibody-like binding specificity) and fragments of said cross-reactive ligands, perhaps produced using one or more of the techniques of recombinant DNA technology referred to and discussed above. Also included are single domain ligands (mAbs) as described in WO 90/05144 (herein incorporated by reference).

Using standard techniques, it is possible to use a ligand, *e.g.*, antibodies of the present invention and derivatives thereof, in immunopurification of a binding partner antigen. Techniques for immunoaffinity column purification are well known, see for instance "Current

Protocols in Immunology," ed. J. E. Coligan et al, John Wyley and Sons, Unit 8.2 (herein incorporated by reference). In fact, it should be possible to use an immunoaffinity column to isolate cross-reactive ligands as discussed above, without needing to isolate the antigens themselves. A first round of immunoaffinity purification uses a ligand, *e.g.*, MT3, 6G3, etc. mAb to remove from a sample the antigen-containing binding partner, which may then be used in the column to select, from a heterogeneous population of ligands, those ligands which are cross-reactive with the MT3 mAb, 6G3 mAb, etc. and recognize the same binding partners.

A binding partner isolated using the ligand, *e.g.*, the MT3, 6G3, etc. mAb may be used to select cross-reactive ligands from a repertoire or heterogenous population of antibodies generated by a whole variety of means. One way is to select monoclonal antibodies and cell lines producing them by the standard hybridoma techniques. Also provided by the present invention are immortalized cells, *e.g.*, hybridomas producing said cross-reactive ligands.

Another way of selecting ligands which are cross-reactive with a ligand such as the MT3 mAb or 6G3 mAb is to use the methods for producing members of specific binding pairs disclosed in WO 92/01047 (Cambridge Antibody Technology Limited and MRC/McCafferty et al, herein incorporated by reference). This publication discloses expression of polypeptide chain components of a genetically diverse population of specific binding pair members, such as antibodies, fused to a component of a secreted replicable genetic display package (RGDP), such as a bacteriophage, which thereby displays the polypeptide on the surface. Very large repertoires of displayed antibodies may be generated, and screened by means of antigen binding to obtain one or more antibodies of interest, along with their encoding DNA. DNA encoding for a polypeptide displayed on the surface of an RGDP is contained within the RDGP and may therefore be easily isolated and cloned for expression. The antibody repertoire screen may of course be derived from a human source.

Obviously, once one has an immortalized cell line, *e.g.*, a hybridoma, or an RGDP containing DNA encoding at least a polypeptide component of a binding ligand, one skilled in the art is in a position to obtain (according to techniques well known in the art) the entire nucleotide sequence encoding the ligand, *e.g.*, the mAb secreted by the cell. Therefore, the present invention also encompasses primary nucleotide sequences which encode the ligands, *e.g.*, mAbs as defined above, together with fragments of these primary sequences and secondary nucleotide sequences comprising derivatives, mutations and hybridizing partners of said primary nucleotide sequences.

These nucleotide sequences may be used in a recombinant system to produce an expression product according to standard techniques. Therefore, the present invention includes vectors (cloning and expression vectors) incorporating said nucleotide sequences, transformed cells incorporating said vectors and expression products produced by use of a recombinant system utilizing any such vectors or transformed cells.

The production of fusion proteins is also contemplated. See, for instance, Stamenkovic et al, "The B Lymphocyte Adhesion Molecule CD22 Interacts with Leukocyte Common Antigen CD45RO on T Cells and α2-6 Sialytransferase, CD75, on B Cells," CELL, Vol. 66, pp. 11-33-1144 (1991), herein incorporated by reference.

The present invention also includes methods for expressing a ligand, *e.g.*, a mAb, derivative, functional equivalent or fragment thereof, which comprises using a nucleotide sequence, vector or transformed cell as defined above.

More specifically, MT3 and 6G3 which are mAbs directed against the human CD45RB antigen will bind to an epitope on CD45RB in human cells expressing CD45RB. This epitope may then be purified, for instance utilizing an immunoaffinity column (as discussed), and partially or wholly sequenced, for instance using repeated rounds of Edman degradation.

EXAMPLE 1: Murine Monoclonal Antibody to CD45RB

Murine monoclonal antibody to human CD45RB is produced by using conventional techniques, essentially as described by Kohler and Milstein in Nature 256: 49. Female BALB/C mice (20-25 g) each receive 100μg of antigen containing human CD45RB, *e.g.*, Hodgkin cell line DEV (publicly available), by i.p. injection. (Alternatively, the antigen may comprise murine cells which have been transformed to express human CD45RB). After 2 weeks a second booster injection comprising 50μg of the antigen is administered, again by i.p. injection. The presence of antibodies reactive to the antigen in the animals' blood serum is confirmed by immunohistologic screening. Mice displaying maximum blood serum levels of CD45RB antibody receive another booster injection comprising 20μg of antigen. Four days later, they are sacrificed and their spleen cells are isolated and fused with a suitable myeloma line, *e.g.*, myeloma X63 (publicly available). The resulting hybridoma are cultured and selected for expression of antibody having a high affinity to CD45RB.

A hybridoma line producing murine monoclonal antibody to human CD45RB is the MT3 hybridoma line, which was deposited on March 29, 1993 under the Budapest Treaty with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852/U.S.A.

A second hybridoma cell line, which produces rat monoclonal antibody to murine CD45RB, is HB220 (now designated MB23G2). This cell line has been deposited with the ATCC and is available by purchase from the ATCC.

A third hybridoma cell line (deposited with the ATCC as HB-11873), produces antibodies of the invention (6G3 mAb). This hybridoma cell line was produced by the fusion of myeloma cell line SP2/O and spleen cells from a mouse immunized with human large cell B cell non-Hodgkin lymphoma cell line VEF. The resulting clones were screened by an immunoperoxidase

procedure on frozen tissue sections of human tonsil and rhesus monkey spleen. Clone 6G3 was selected because of the high reactivity of 6G3 mAb with subsets of T and B lymphocytes in both tissues. The antibody reactivity of 6G3 was characterized as anti-CD45RB by its selective reactivity with human CD45RB expressing transfectants and by the characterization of the molecular weight of the antigen immunoprecipitated by 6G3 as three bands with molecular weights of 220, 204 and 190 kD. The reactivity of the antibody could be abolished by pretreatment of tissues, cells or blots with neuraminidase, indicating the sialic acid dependence of the antigen.

A fourth hybridoma cell line, HB223, produces analogous monoclonal antibodies to MB23G2; it is also deposited and available through the ATCC.

EXAMPLE 2: Chimeric Monoclonal Antibody to CD45RB

a) Cloning of the gene encoding the variable domain of the heavy chain

The genomic DNA of the desired hybridoma, in this example the MT3 or 6G3 hybridomas of Example 1, and of the parental myeloma cell lines of the hybridomas (myeloma X63 or SP2/O) is isolated and digested with EcoRI. Each digested DNA is then fractionated on the same agarose gel. After migration, the agarose gel is analyzed by Southern blot using as probe a ^{32}P labelled 0.7kb XbaI-EcoRI DNA fragment which encodes the murine heavy chain enhancer Eμ (Heinrich et al, J. OF IMMUNOL. (1989) 143: 3589) to identify the desired variable heavy chain fragment, *i.e.*, the desired fragment is present in the MT3 and 6G3 hybridomas but not in the X63 or SP2/O myelomas. Further purification of this fragment is then carried out by preparative agarose gel electrophoresis.

DNA fragments of the same size as the desired fragment are cloned in the EcoRI restriction site of bacteriophage ZAP (Stratagene). Using the probe described above, the

recombinant phages are screened and clones selected which hybridize to the probe. The DNA inserts of the selected clones are amplified on phage plate lysate by polymerase chain reaction (PCR) using as primers, a first oligonucleotide encoding the murine J_{κ} gene and a second oligonucleotide encoding the beginning of the MT3 or the 6G3 heavy chain. The DNA fragments obtained from each of the selected clones are analyzed by Southern blot using as probe an oligonucleotide encoding a portion of the $E\mu$ probe described above.

b) Construction of a chimeric heavy chain gene

The EcoRI fragment (comprising the gene of the MT3 or 6G3 heavy chain variable domain (including the promoter and the enhancer)) is obtained by digestion of the DNA of one of the phage clones selected in step a) is then cloned into the EcoRI restriction site of the eukaryotic expression vector pSV2 neo-human γ_1, constant part (Heinrich et al, supra). Following propagation of the resulting plasmid, the nucleotide sequence of the gene encoding the MT3 or 6G3 heavy chain variable domain is redetermined to exclude the possibility that a mutation in this gene has occurred.

c) Cloning of the gene encoding the variable domain of the light chain

The genomic DNA of the MT3 or 6G3 hybridomas and of the parental cell lines X63 or SP2/O is isolated and digested with EcoRI. Each digested DNA is then fractionated on the same agarose gel. After migration, the agarose gel is analyzed by Southern blot using as probe a ^{32}P-labelled DNA fragment comprising the five mouse J_{κ} genes and the mouse C_{κ} gene. Size fractionated EcoRI fragments corresponding in size to the desired MT3 or 6G3 light chain variable domain are cloned in phage EMBL4 (Stratagene).

A clone containing the DNA fragment encoding the MT3 or 6G3 light chain is identified by screening the recombinant phage clones with the probe described immediately above. The

desired DNA fragment is then subcloned into the EcoRI - XbaI site of pGEM4 (Promega) and its sequence determined.

d) Construction of a chimeric light chain gene

An XbaI - XbaI fragment containing the sequence encoding the murine heavy chain enhancer (Heinrich et al; supra) and a HindIII - SphI DNA fragment containing the sequence for the human κ constant part (huCκ) are cloned together into phage mp18 (Stratagene). Site-directed mutagenesis is performed on the resulting recombinant phage to disrupt the HindIII site in the desired coding region and followed by digestion with EcoRI and HindIII to generate a DNA fragment containing the sequences for both (Eμ) and (huCκ). After filling in the ends of this fragment, the fragment is subcloned into the blunt-ended EcoRI-BamHI site of pSV2-DHFR to generate pSV2-DHFR-Eμ-huC$_\kappa$. The plasmid pSV2-DHFR is obtained by replacing the BamHI - HindIII fragment of pSV2-neo with a BamHI - HindIII fragment encoding the dihydrofolate reductase gene.

Lastly, an EcoRI-XbaI DNA fragment containing the MT3 or 6G3 light chain sequence is isolated from the recombinant pGEM4 plasmid of step 3 and subcloned into pSV2-DHFR-Eμ-huC$_\kappa$ to generate pSV2-DHFR-Eμ-huC$_\kappa$-MT3$_L$ or pSV2-DHFR-Eμ-huCκ-6G3$_L$.

e) Expression of chimeric antibody

The plasmids obtained in steps b) and d) are co-transferred into the mouse myeloma cell line SP2/0 (ATCC CRL 1581) by electroporation using a gene pulser apparatus from Biorad. This technique is known to create stable transfectants at a high frequency. The SP2/0 cell line fails to produce endogenous heavy and light chains and is sensitive to Geneticin (G 418) at a concentration of 0.8 mg/l.

SP2/0 cells are grown in the usual growth medium (RPMI + 10% FCS 5X10^{-5} β-mercaptoethanol) harvested in the log phase of growth and washed with the electroporation

buffer (Bio-Rad). Cell concentration is adjusted to $2x10^7$ cells/ml. To 0.8 ml of the cell suspension is added 15-20μg of each plasmid. The mixture is placed on ice and left to stand for 10 min. Then the cells are subjected to an electrical pulse (280 Volt; 25 °F) and again left to stand for 15 min. Cells are transferred to the usual growth medium and incubated at 37°C in a CO_2 incubator.

After a 3-day incubation, selection for G 418 resistance is started. Cells are re-suspended in fresh medium containing 1.4 mg/ml G 418. The cultures yield growing cells after 10-14 days incubation in the presence of G 418. After the 2-week incubation, supernatants of the confluent cultures are tested for human IgG expression in a sandwich-type ELISA (anti-human κ-light chain/supernatant/anti-human IgG-alkaline phosphatase conjugate).

This test indicates that complete antibody molecules are secreted in all cultures at varying concentrations in the range of 50-500 ng/ml.

To select cells in which the DHFR gene is amplified and which therefore secrete high amounts of the desired antibody, two selection procedures for Methotrexate (MTX) resistance are carried out as described below. For this purpose, the G 418 resistant cell pools are each divided and amplification is preformed either according to procedure A (MTX increase by a factor of 2 or 2.5) or procedure B (MTX increase by a factor of 5).

Procedure A	Procedure B
100nM MTX	200nM MTX
250nM MTX	1μM MTX
500nM MTX	5μM MTX
1μM MTX	25μM MTX
2.5μM MTX	100μM MTX
5μM MTX	

Procedure A	Procedure B
10μM MTX	
25μM MTX	
100μM MTX	

Each amplification step comprises inoculating the cells at a density of 2x10^5 cells/ml in the usual growth medium supplemented with G 418 at 1.4 mg/ml and with MTX at the concentration of choice. After 72 hour incubation, cells and the supernatant are separated. Antibody secretion is monitored either by ELISA or by HPLC using a protein A column. Most of the pools reach a maximum of specific antibody production at a certain MTX concentration. The best producing pools are cloned by limiting dilution. Out of several hundred analyzed clones, 15 best producing clones are selected. Productivity of the clones ranges from 30 to 50 mg mAb/10^9 cells in 72 hours.

The antibody is purified from a culture supernatant by elution on a protein A affinity column.

The compounds and pharmaceutical compositions of the invention (the Compounds) are useful in immunomodulation, especially immunosuppression, *e.g.*, in the following indications:

a) Treatment and prevention of organ or tissue allo-or xeno-transplant rejection, *e.g.*, for the treatment of recipients of , *e.g.*, heart, lung, islet cells, bone marrow, chromaffin or dopamine producing cells, combined heart-lung, liver, kidney, pancreatic, skin, small bowel, or corneal transplants. They are also indicated for the prevention of graft-versus-host disease, such as sometimes occurs following bone marrow transplantation.

b) Treatment and prevention of autoimmune disease and of inflammatory conditions, in particular inflammatory conditions with an etiology including an autoimmune component such as arthritis (for example rheumatoid arthritis, arthritis chronica progrediente and arthritis

deformans) and rheumatic diseases. Specific autoimmune diseases for which the compounds of the invention may be employed include, autoimmune hematological disorders (including, *e.g.*, hemolytic anemia, aplastic anemia, pure red cell anemia and idiopathic thrombocytopenia), systemic lupus erythematosus, polychondritis, scleredema, Wegener granulomatosis, dermatomyositis, chronic active hepatitis, myasthenia gravis, psoriasis, Steven-Johnson syndrome, idiopathic sprue, autoimmune inflammatory bowel disease (including, *e.g.*, ulcerative colitis and Crohn's disease) endocrine ophthalmopathy, Graves disease, sarcoidosis, multiple sclerosis, primary biliary cirrhosis, juvenile diabetes (diabetes mellitus type 1), uveitis (anterior and posterior), keratoconjunctivitis sicca and vernal keratoconjunctivitis, interstitial lung fibrosis, psoriatic arthritis, glomerulonephritis (with and without nephrotic syndrome, *e.g.*, including idiopathic nephrotic syndrome or minimal change nephropathy) and juvenile dermatomyositis.

c) Treatment of leukemias characterized by over-proliferation of T-lymphocytes, including virally induced leukemias, *e.g.*, HTLV-1-induced leukemia.

The compounds of the invention are shown to prevent mice from rejecting kidney transplants and induces long term survival. The compounds of the invention are also shown to reverse and prevent rejection of organ transplants in rodents and primates.

It is also contemplated that the CD4RB antibody may be given alone with standard immunosuppressant or anti-inflammatory agents. These would include Cyclosporin, FK-506, Leflunomide, Rapamycin, mycophenolate mofetil, Deoxyspergualin, corticosteroids, anti-lymphocyte globulin, OKT-3 and the like, and others. Use of the CD45RB antibody is expected to reduce the dosage requirements for such drugs and thereby to reduce undesired side effects. The Compounds may also be used in combination with other monoclonal antibodies or other compounds specifically recognizing particular lymphocyte sub-populations, *e.g.*, CD25 mAbs, CTLA4-Ig fusion peptide, etc.

The Compounds have a particular advantage over conventional immunosuppressants and anti-inflammatory drugs in that they are capable of inducing long term antigen-specific tolerance to an antigen when the subjects are treated with the Compounds when first exposed to the antigen. For example, we have shown that in mice surviving over 100 days following a kidney allograft, skin grafts syngeneic with the donor kidney were tolerated, while skin grafts allogeneic with both the recipient and the donor kidney were rejected. It is believed that T-cells having a receptor for the antigen are anergized, and that this T-cell clone is effectively deleted. That is, it is not enough that the T-cell recognize a foreign protein, it requires the presence of a second signal, *i.e.*, that of CD45. If CD45 is not recognized the T-cell clone is deleted. For example, we have shown that in a mouse kidney transplant model, allograft tolerance following initial treatment with anti-CD45RB monoclonal antibody persists indefinitely, with survival well in excess of 100 days.

<u>EXAMPLE 3: *In vivo* prevention of the rejection of kidney transplants</u>

In this experiment a right nephrectomy was performed on 18 mice at the same time an allograft (kidney transplant from a different strain of mouse) was performed. A contralateral nephrectomy was followed on the seventh postoperative day (POD 7), so that from that point on, the animals relied only on the allografted kidney. Nine of the mice were treated with 50μg of a mixture of rat anti-mouse CD45RB monoclonal antibodies produced from cell lines HB220 and HB223 by iv for the first two days (POD 0 and POD 1) followed by 100μg of each antibody intraperitoneally for 9 days (POD 2 to POD 10). Of the nine control animals which did not receive the anti-CD45RB antibodies, seven were dead three days after the second kidney was removed, and the remaining two exhibited severe rejection one week later. Of the nine animals treated with the anti-CD45RB antibodies, there were three deaths due to surgical complications

unrelated to any immune response, but remarkably, the remaining six animals survived long term (*e.g.*, over 100 days) without any further treatment and without any evidence of allograft rejection. In a third group of 10 untreated isograft recipients, the incidence of death due to surgical complication was the same. There was no significant difference between the serum creatinine levels of the allograft group receiving monoclonal antibody and the isograft group, indicating that the kidneys in both groups were functioning normally.

EXAMPLE 4: Reversal of the rejection of kidney transplants in mice

In this experiment a right nephrectomy was performed on 10 mice at the same time an allograft (kidney transplant from a different strain of mouse) was performed.

All ten of the animals were observed for five days without immunosuppression therapy. These animals were known to be experiencing severe rejection at this stage because sacrificed control animals, also subjected to a nephrectomy and an allograft kidney transplantation, exhibited severe rejection on day 5.

On POD 5, four of the animals were given three daily 25 μg doses, intraperitoneally, of anti-CD45RB antibody (a mixture of monoclonal antibodies from cell lines HB220 (anti-CD45RB MB23G2 mAb) and HB223 (anti-CD45RB MB4B4 mAb)) for the next three days. All four of the animals experienced rapid reversal of their rejection symptoms, including a return to normal levels of creatinine, and lived greater than 100 days. The untreated animals died by day nine due to organ rejection. Table I summarizes the results of this experiment:

TABLE I

REVERSAL OF THE REJECTION OF KIDNEY TRANSPLANTS IN MICE

MOUSE	THERAPY	SURVIVAL DAYS	CAUSE OF DEATH
1	NONE	8	REJECTION/UREMIA
2	NONE	9	REJECTION/UREMIA
3	NONE	8	REJECTION/UREMIA
4	NONE	9	REJECTION/UREMIA
5	NONE	9	REJECTION/UREMIA
6	NONE	9	REJECTION/UREMIA
7	CD45RB	>100	--
8	CD45RB	>100	--
9	CD45RB	>100	--
10	CD45RB	>100	--

This data with respect to reversal is significant in confirming that the antibody therapy is truly effective in suppressing an immune response. Treatment and cures are accomplished with antibody therapy.

EXAMPLE 5: Confirming results using MB23G2 and MB4B4 separately

Recipient Balb/c (h-2d) mice had the right kidney removed before receiving a transplanted kidney from donor C57B1 (h-2b) mice. A left native nephrectomy was subsequently performed on day 7. There were four groups of animals. Thirteen received isografts, 17 received allografts with no immunosuppression (vehicle control), 44 received allografts and were given two doses of purified rat anti-mouse CD45RB mAb MB23G2 1mg/kg (30μg) intravenously on days 0 and 1, and 16 received allografts but were treated with two doses of purified rat anti-

mouse CD45RB mAb MB4B4 1mg/kg (30μg) intravenously on days 0 and 1. No further immunosuppression was given.

As expected, the MB23G2-treated animals had prolonged survival compared to the untreated group ($p < 0.002$) (see **Fig. 1**) and was comparable to the isograft group. Remarkably, the anti-CD45RB MB4B4 mAb was no better than the vehicle alone at preventing rejection. Both MB23G2 and MB4B4 are IgG2a but a difference exists between them. Both mAbs bind to Balb/C leukocytes as assayed by FACS. However, MB23G2 binding is inhibited by neuraminidase, while binding of MB4B4 is not affected by such treatment. **Figure 2** shows the serum creatinine levels in animals from each group at the time of sacrifice, or beyond day 100 for the long-term survivors. There were no differences between the isograft and MB23G2-treated groups, while the untreated and MB4B4-treated animals died from uremia. Therefore, the glycosylated epitope for MB23G2 is either involved in, or is near to, sites involved in the biochemical activity of CD45RB. The non-glycosylated epitope for MB4B4 appears to be non-critical for CD45RB activity.

Immunoperoxidase microscopic studies were performed on renal allografts at 7 days in three groups of mice: untreated, MB4B4-treated, and MB23G2-treated. Sections were stained with rat anti-mouse mAb reactive with mouse CD3, CD4, CD8, CD45RB and Ia. Slides were evaluated in a masked manner with respect to aggregates and diffuse infiltrates as described in Ibrahim et al., TRANSPLANTATION Vol. 59, pp. 724-728, herein incorporated by reference. The numbers of cells in the diffuse infiltrates were counted in ten (x 400) high power fields (HPF) in each section of 5 mice per group and the data are shown in Table II below.

TABLE II
DIFFUSE CELLULAR INFILTRATES IN KIDNEY ALLOGRAFTS AT 7 DAYS
(MEDIANS/HPF)

Phenotype	mAb	Untreated	MB4B4-Treated	MB23G2-Treated
CD3	KT3	33	38	22*
CD4	GK1.5	10	11	9
CD8	3.155	27	26	13*
CD45RB	MB23G2	12	10	9

* $P < 0.05$. Statistically significant differences between MB23G2-treated and nontreated groups and between MB23G2-treated and MB4B4-treated groups.

Interestingly, differences between the three groups of mice became evident after separately counting cells in aggregates and diffuse infiltrates. Staining for Ia was clearly less in the MB23G2-treated allografts than in the other groups, but due to positive staining of other interstitial cell types the numbers of lymphocytes could not reliably be quantified. The aggregates contained high numbers of CD4+ and CD8+ cells in all three groups. While the numbers of CD4+ and CD45RB+ cells in the diffuse infiltrates were approximately equivalent in all three groups (Table II, second and fourth rows) the numbers of CD3+ cells and CD8+ cells in the diffuse infiltrates were statistically different between MB23G2-treated and the other groups (Table II, first and third rows). Thus, the MB23G2-treated animals demonstrated an elevated CD4:CD8 ratio compared to MB4B4-treated and untreated animals. Remarkably, few of the infiltrating cells were CD45RB positive, a particularly notable finding considering that the CD45RB mAb MB23G2 could reverse acute rejection.

To assess the possibility of antigen specific tolerance, skin transplants were performed on 13 animals which had maintained a kidney transplant beyond 100 days after receiving 2 doses of MB23G2 mAb at the time of renal allografting. Each animal received full thickness skin

allografts from a C57B1/6 mouse (isogeneic with the donor of the renal allograft) and a control skin transplant: 9 received a Balb/C isograft and 4 received a CBA allograft (third party donor). No further immunosuppression was given. Of the 13 animals with kidney specific tolerance, there was a subset of 4 which demonstrated donor alloantigen specific tolerance since they did not reject the C57B1/6 skin. All 4 animals rejected the third party CBA skin, while all 9 Balb/C isografts survived indefinitely. No renal allograft rejection was stimulated by the skin transplants.

To determine whether MB23G2 mAb could reverse acute rejection, seven allografts were performed as described above, but no immunotherapy was administered until day 4. Untreated allografted kidneys demonstrated rejection at this time. The treated animals received MG23G2, 1.5 mg/kg (50 μg) i.v. daily on days 4, 5 and 6 and no further therapy thereafter. Three animals died of ureteric complications in the MG23G2 treated group - the graft histology did not show rejection at the time of death on days 8, 9 and 25. All the animals had their rejection reversed and the remaining 4 survived > 60 days with a normal serum creatinine.

The pharmacologic effects of MB23G2 on the peripheral blood in mice were assessed using multiparameter FACS analysis. Mice were treated with 30 μg of MB23G2 mAb intravenously on two consecutive days. As shown in **Fig 3A**, MB23G2 induced a significant depletion of circulating lymphocytes which returned to normal one week after stopping the mAb. MB23G2 bound to almost all the remaining circulating T and B lymphocytes (**Fig. 3B and 3C**). FACS analysis revealed no excess MB23G2 antibody in the plasma by day 8. FACS analysis of the spleen demonstrated that the administered therapy penetrated the lymphoid tissue 24 hours after the second dose of MB23G2. A FACS inhibition assay did not reveal sensitization of any of the mice to the MB23G2 mAb up to 2 weeks after therapy.

Since CD45 is a protein tyrosine phosphatase, tests were designed to show that induction of allograft tolerance by MB23G2 mAb is related to an alteration in tyrosine phosphorylation of T cell substrates necessary for signal transduction to occur.

EXAMPLE 6: Mechanism of tolerance induction by CD45RB monoclonal antibody: increased tyrosine phosphorylation of phospholipase C-γ1 and decreased expression of inflammatory cytokines.

Murine T cell hybridoma A1.1 cells were stimulated with the CD3 mAb 2C11 in the presence or absence of MB23G2 or MB4B4 mAb. The cells were lysed in Brij 96 and the phosphotyrosine-containing proteins were immunoprecipitated with anti-phosphotyrosine mAb PY72 (a gift from Dr. G. Mills and Dr. B. Sefton. The immunoprecipitated proteins were extracted and separated on 10% SDS-polyacrylamide gels, transferred electrophoretically to PVDF membranes and submitted to an immunoblotting procedure (see Lazarovits et al., J. IMMUNOL., Vol. 153, pp. 3956-3966 (1994), herein incorporated by reference) using the anti-phosphotyrosine mAb 4G10 (a gift from Dr. B. Druker).

It was found that there was an augmentation of tyrosine phosphorylation of a 145 kDa substrate in the presence of the CD45RB MB23G2 mAb. The MB4B4 mAb did not alter the tyrosine phosphorylation of this substrate. The identity of this 145 kDa band as PLC-γ1 was confirmed by stripping the 4G10 mAb from the blots and reprobing with monoclonal antibody to phospholipase C-γ1 (PLC-γ1) (Upstate Biotech, Lake Placid, NY). Thus, in the presence of MB23G23, substantially more tyrosine phosphorylated PLC-γ1 could be identified than in its absence, while MB4B4 did not alter the amount of (PLC-γ1) which could be immunoprecipitated. The increased tyrosine phosphorylation of (PLC-γ1) has been noted by

Gajewski et al (PROC. NATL. ACAD. SCI. USA, Vol. 91, pp. 38-42 (1994)) to be a property of anergic T cells.

Since gene activation is a consequence of signal transduction in T cells, experiments were performed to investigate whether the MB23G2 mAb could alter the expression of cytokine genes *in vivo* known to be increased in rejecting allografts. It is generally believed that a so-called TH1 cytokine profile (IL-2, γ-interferon) is associated with rejection, while a TH2 phenotype (IL-4, IL-5, IL-6, IL-10) may be associated with non-responsiveness (see T. R. Mosmann, et al., J. IMMUNOL., Vol. 136, pp. 2348-2357 (1986)).

To examine gene expression in mouse renal allografts, steady state levels of specific mRNA transcripts were assessed by Northern Blot analysis using ^{32}P-labelled cDNA probes. Gene expression in four groups of animals was examined: isografts on postoperative day 7, allografts from untreated animals on postoperative day 7, and allografts from MB23G2-treated animals on postoperative day 7 and day 28. No specific pattern of IL-1, IL-2, IL-4, IL-5, IL-6 or IL-10 was detected. However, there was a selective decrease on day 28 in mRNA transcripts for γ-interferon and tumor necrosis factor α compared to untreated allografts. There was no difference noted on day 7. Interestingly, intercellular adhesion molecule-1 (ICAM) mRNA was also decreased in the MB23G2-treated animals on day 28, with no difference observed on day 7. Thus, the MB23G2 therapy may induce tolerance in part by inhibiting the expression of inflammatory cytokines because of interference with the signal transduction cascade.

In yet other experiments, use of anti-CD45RB antibodies were found to be effective when administered to primates.

EXAMPLE 7: Prevention of organ rejection in primates and reversal of organ rejection

Renal allografts were performed on two Cynomolgous monkeys using a CD45RB monoclonal antibody (which binds to a neuraminidase sensitive epitope), as an immune suppressor and the details are set forth below:

Detailed Experimental Procedures:

1. Animal care:

The animals were housed in the University of Western Ontario primate facility. They were provided with squeeze cages which allow for drug injections and sample collection without having to anesthetize the animal, thereby reducing stress. They were maintained on standard monkey feed, and other foods for diversity. They were allowed regular exercise in the exercise cage. The animal care followed the standard operating procedures for non-human primates provided by veterinary services.

Animals were typed for blood groups. On arrival, the animals were rested for at least 2 weeks. The animals were anesthetized with atropine and ketamine for physical examination, including inspection for oral B virus, TB testing, and de-wormed with Ivernectin 2828. Animals fasted the night prior to any anesthetics.

2. Kidney transplantation:

1. Donor procedure:

Two donor animals were injected with ketamine, taken to the OR, intubated and put on insofluorane/nitrous oxide. A three stage surgical prep is used. After a midline incision of the two, the left renal artery, vein and ureter were carefully isolated and divided. Grafts were ex-vivo perfused and stored in 4°C University of Wisconsin solution. The wounds were closed and the animals returned to the cage to recover from the anesthetic. 200 - 300 ml of saline were

given by continuous i.v. during the surgery. During the surgery, the animals were kept warm using a heating lamp, heated saline and heating pad, etc.

Postoperative care followed Standard Operating Procedures. Briefly, the animals remained on a warm water blanket and under a heating lamp for 24 hours. Buprenorphine was given q6h after surgery for 24 hours. The animals were monitored daily. The well recovered donor animals are used as the recipient in future transplantations. The interval between the two surgeries is at least two weeks.

2. *Recipient procedure:*

Recipient was anesthetized and prepared preoperatively as described for the donor. After a midline incision, abdominal aorta and inferior vena cava were exposed. End-to-side anastomoses were performed between the donor renal artery and the recipient aorta, as well as between the donor renal vein and the recipient inferior vena cava. The donor ureter was sutured to the recipient's bladder. The right kidney was removed and the wound was closed.

3. *Post-operative care:*

The post-operative care is the same as described for the donor. Animals were monitored continually post-operatively for at least 24 hours, more if necessary. They were monitored closely (*i.e.*, several times per day) until feeding and grooming normally. Thereafter, they were monitored at least daily when they received their monoclonal antibody.

Animals were given 4 mg of anti-CD45RB 6G3 mAb 4 i.v. daily for 7 days. The outcome of kidney grafts was measured by percutaneous biopsy weekly and blood creatinine levels twice per week. For these procedures animals were anesthetized with ketamine. Criteria for early euthanasia would include lethargy, lack of grooming or feeding, significant weight loss (> 20%) and renal failure (elevated creatinine levels).

As discussed above, the recipient animals received 4mg (1mg/kg) of anti-CD45RB 6G3 mAb post operatively for 7 days. There were no side effects associated with such infusions. Both animals survived normally until day 16 when each experienced an acute rejection crises. The first animal was euthanized 2 days later on day 18. The second animal was re-treated with 4 mg/kg (16 mg) of anti-CD45RB 6G3 mAb. This therapy was given daily by iv for four days. Remarkably the rejection crises completely reversed as the animal was observed to resume normal activities and creatine levels were observed to decrease from a "crises" level of 738 μmol/L to 366 μmol/L. The animal remained well until day 36 when another rejection crises developed. The animal was than euthanized. Histology of the allograft revealed that there was profound endotheliitis on post-operative day 15 just before additional therapy was administered leading to reversal. A biopsy of the allograft of this animal was performed on post-operative day 23 revealing the endotheliitis had cleared.

It is known that control animals will die by day 10 if therapy is not administered in this type of model. (Lazarovits, et al, "A Monoclonal Anti-Act I Reacts with a Late Lymphocyte Activation," KIDNEY INTER. 25:344).

The data suggests two conclusions:

[1] Because both monkeys lived past the known date of controls, it is shown the therapy of the invention exhibits significant graft-survival in a primate.

[2] Even more dramatic is the observation that one can reverse acute rejection with anti-CD45RB monoclonal antibodies.

EXAMPLE 8: Additional Monkey Experiments

Two additional Cynomolgus monkeys (#3 and #4) have received renal allografts and have been treated with the CD45RB monoclonal antibody 6G3 as the sole form of

immunosuppression. Blood grouping was performed to control for ABO compatibility and major histocompatibility complex profiles were obtained using PCR based DNA typing to confirm that allogeneic renal transplants were being performed.

On day zero, a nephrectomy was performed in the recipient animal and the renal allograft was performed. On day seven the second native kidney was removed and from that point on the animal relied on its transplanted kidney. Animals which do not receive immunosuppression or which receive ineffective immunosuppression will reject at a mean of ten days +/-two days (Lazarovits, et al 1984. "A Monoclonal Antibody Anti-Act I Reacts with a Late Lymphocyte activation," KIDNEY INTERN. 25:344).

Monkey #3

This animal was treated with 6G3 antibody 2 mg/kg/day (8 mg) x 7 days and then 6 more doses given on Monday, Wednesday and Friday for each of the next two weeks was to be given. Thus 8 mg of 6G3 antibody was planned to be given over three weeks. The animal developed rejection on day 14 and was euthanized.

Monkey #4

This animal received the same therapy as monkey #3. That is 8 mg of 6G3 antibody was given intravenously for 13 doses over three weeks. This animal has done remarkably well and continues to be alive beyond 70 days. No rejection has been diagnosed.

Thus, animals #3 and #4 treated with 6G3 have had significantly prolonged allograft survival which is illustrated in **Fig. 4**. Monkey #2 of Example 8 is of particular interest because the antibody successfully reversed acute rejection which was predicted by the mouse kidney transplant experiments. Additional experiments are underway to try to determine the cause for relatively early graft failure in monkeys 1 and 3, although both of these animals also had significantly prolonged allograft survival.

EXAMPLE 9: *In vivo* prevention of the rejection of heart transplants.

Heterotopic heart transplants from C57B1/6 mice into BALB/C donors were performed essentially as described in R.L. Kirkman et al (1985) Transplantation 40: 719-722. Seven of the mice received 30 μg iv of rat anti-mouse CD45RB mAb MB23G2 on days 0 and 1 following heart transplantation. Four other mice received 30 μg i.v. on days 0 and 1 and 100 μg ip of rat anti-mouse CD45RB mAb MB23G2 daily on days 2 to 11 following heart transplantation. Fourteen control mice received no antibody. Survival of the heterotopic graft was determined by whether the heart was beating and rejection was confirmed by histological analysis. All of the control mice had rejected their hearts by day 14 post-operation, with a mean survival time of 9 days. Mean survival time of the hearts in the group receiving antibody for two days only was 20 days and mean survival time of the hearts in the group receiving antibody for 11 days was 34 days. Table III summarizes the results from this experiment.

TABLE III
MOUSE CARDIAC ALLOGRAFTS

Groups	Number	Survival (days)	Mean
Untreated	14	8,8,9,9,9,9,9,9,9, 10,11,11,11,14	9
CD45RB mAb 30 μg D0,D1	7	16,16,17,22,24,23,24	20
CD45RB mAb 30 μg IV D0,D1 AND 100 μg ip x 9 days	4	15,30,38,38	34

Additional evidence for potential utility of anti-CD45RB was demonstrated in the following studies:

EXAMPLE 10: *In vivo* prevention of the rejection of pancreatic islet allograft transplants.

Pancreatic islet allografts were transplanted under the kidney capsule from CBA/J donors into streptozotocin-treated BALB/C recipients essentially as described in M.C. Fabian et al (1993) Transplantation 56: 1137-42. Five control mice received no antibody while eleven mice received 30 μg i.v. of rat anti-mouse CD45RB mAb MB23G2 on days 0 to 1 post-operation. Rejection was defined as onset of glycosuria. All islet allografts from control mice had been rejected by day 24 with a mean rejection time of 17 days. Antibody-treated mice showed a mean rejection time of 34 days with two mice having no signs of rejection at day 50 when the experiment was stopped.

Table IV summarizes the result from this experiment.

TABLE IV
MOUSE PANCREATIC ISLET ALLOGRAFTS

Groups	Number	Survival (days)	Mean
Untreated	5	12,12,15,24,20	17
CD45RB mAb 0 μg iv D0,D1	11	23,32,20,30,30 >50,>50,21,23,47,50	34

EXAMPLE 11: Induction of Xenograft Tolerance in Rat to Mouse Transplant Models

To assess whether anti-CD45RB monoclonal antibody could prevent xenogeneic renal graft rejection, orthotopic kidney xenografts were performed in BALB/c mice with Lewis rats as donors. Five groups of recipients were studied: no treatment (Controls), cyclosporine (CsA) treatment (5 mg/kg S.C. daily), splenectomy (Spl), cyclophosphamide (CyP) treatment (20 mg/kg on POD 0, 2, 4 & 7), MB23G2 mAb treatment (100 μg daily x 11 days, I.P.), and combined treatment with MB23G2 mAb (100 μg x 11 days) and CyP (20 mg/kg I.V. on POD

0, 2, 4 & 7). As shown below in Table V, animals treated with MB23G2 mAb and CyP had a significantly longer median survival time than animals treated with mAb alone or CyP alone, demonstrating that CD45RB mAb and CyP have a synergistic effect on prolonging renal xenografts in the mouse.

TABLE V

RAT-TO-MOUSE KIDNEY XENOGRAFTS

Group	n	Treatment	Survival (days)	Median Survival (Days)
Control	6	None	6(4), 7, 16	6
CsA	3	Cyclosporine	4, 6, 8	6
Spl	5	Splenectomy	4,5,6,7,11	6
CyP	8	Cyclophosphamide	10, 18, 23, 24, 28, 49, 58, >100	26
mAb	6	MB23G2	4, 6, 8, 8, 11, 13	8
mAb+CyP	9	CyP + MB23G2	9, 11, 23, 24, 70, 76, >100(3)	70*

*P<0.01, the mAb group vs the control group.

The ultimate goal for clinical xenotransplantation is to induce xenograft tolerance, thereby eliminating the continuous use of toxic, high-dose immunosuppression. It was recently demonstrated in a hamster heart to mouse xenograft model that the combination of pulse therapy with CyP and continuous CsA treatment induced prolonged graft survival. See Hasan et al., "Evidence That Long-Term Survival of Concordant Xenografts Is Achieved By inhibition Of Antispecies Antibody Production," TRANSPLANTATION, Vol 54, pp. 408-413 (1992). However, when the cyclosporine therapy was stopped, the xenografts were rejected within a

short period of time, *i.e.*, the median survival time was less than three weeks. Thus, therapy with CyP and CsA was unable to induce xenograft tolerance.

In contrast, as shown in Table V above, xenografts in animals treated with CD45RB mAb and CyP continued to survive several months after cessation of immunosuppressive therapy. Moreover, the long term surviving kidney xenografts treated with mAb and CyP had a normal renal function and a normal pathology at sacrifice on POD 100. These results demonstrate that treatment with CD45RB mAb and CyP can induce functional xenograft tolerance in a rat-to-mouse model. This therapy may be applicable in the prevention of transplant rejection in man.

The invention can include a kit containing pharmaceuticals for administration to patients who have received xenografts. The kit can include CD45RB antibodies in an appropriate pharmaceutical formulation, along with one or more drugs used to suppress rejection induced by pre-existing antibodies. Such drugs could include cyclophosphonamides, Deoxyspergualin and the like.

Anti CD45RB may be useful in management of other expressions of the immune response. One example is graft-versus-host (GvH) reaction. Other examples include autoimmune related diseases such as diabetes, lupus, multiple sclerosis, rheumatoid arthritis and the like that are characterized by inflammation resulting from inappropriate immune responses to self antigens.

EXAMPLE 12: *In vivo* treatment of NOD mice to inhibit the onset of diabetes.

Five female NOD mice were treated with 30 μg iv of rat anti-mouse CD45RB mAb MB23G2 on days 28, 29 and 30 after birth. Five control mice received no antibody treatment. By week 27 all control mice were dead as a result of diabetes. Of the antibody-treated mice 2 died of diabetes while the other 3 remained alive and well until week 35 at which point they

were killed and their pancreas examined histologically. There was no sign of insulitis in any of the three surviving animals.

Table VI summarizes the results from this experiment.

TABLE VI
ONSET OF DIABETES IN NOD MICE

Groups	Number	Survival Without Diabetes
Untreated	5	0 (all dead by 27 weeks)
CD45RB mAb 30μg iv days 28, 29, 30	5	3 > 35 weeks

Additional experiments have been conducted since this preliminary experiment was conducted. The therapy in these experiments comprised administering 100ug of MG23G2 two times per week from 2-35 weeks, at which time all remaining mice were euthanized. The results are shown in Table VII below:

TABLE VII

NOD MOUSE SURVIVAL (WEEKS)

THERAPY	SURVIVAL WEEKS	INSULITIS SCORE* (MEAN)
MB23G2 (N=9)	35(6),20(2),13	0.99
CONTROL (N=12)	34(2),30(2),28,23 18,16,15,14,13,12	1.81

* Insulitis score obtained from 6 additional animals in each group at 15 weeks.

As seen from the data a significantly greater number of animals survived indefinitely, *i.e.*, until the end of the 35 week experiment compared to the controls. The improvement induced by MB23G2 is evident not only in animal survival but also in blood sugar where all of the animals who survived to 35 weeks in the treated group had no evidence of hyperglycemia.

The beneficial effect of MB23G2 was also confirmed by the insulitis score which is a careful histologic assessment of the pancreas. The difference between 1.81 and 0.99 is both biologically and statistically meaningful.

Example 13: Prevention of Experimentally Induced Allergic Encephalomyelitis (EAE) in Mice

EAE is a widely accepted small animal model for multiple sclerosis (MS) in which mice are immunized against myelin basic protein from brain tissue. They develop a clinical syndrome, including tail paralysis, that is considered a model for MS. For this study, on day 1, two groups of mice were injected with myelin basic protein in Freund's adjuvant subcutaneously along with pertussis toxin interperitoneally. One group (six mice) also received 40 μg of MB23G2 mAb interperitoneally on day 1, while the second group (seven mice) served as a control. Pertussis toxin was administered interperitoneally again on day 3. On days 6 and 11, the mAb-treated group received two additional 40 μg doses of MB23G2 for a total of three doses of anti-CD45RB antibody. The animals were then followed clinically for symptoms of the disease (paralysis of the tail and both hind legs), which usually develop within 16 days.

The results were significant. Starting with seven controls and six treated animals, 6 of the controls developed EAE as manifested by tail paralysis. One of the six MB23G2-treated animals died at day 14, although it never manifested any clinical evidence of EAE. The remaining MB23G2-treated animals did not develop tail paralysis or any other clinical evidence of EAE. Thus, the CD45RB mAb MB23G2 was effective in preventing autoimmune disease in this animal model of Multiple Sclerosis.

The pharmaceutical utility of the compounds may be additionally shown, for example, in the following *in vivo* models:

Prevention of skin graft rejection

Four groups of A-strain mice (white) receive skin grafts from C57BL/10-strain mice (black): groups I and II receive prior treatment with rat anti-mouse CD45RB mAb and groups III and IV receive no prior treatment with rat anti-mouse CD45RB mAb. Following the skin graft operation, groups I and III are treated with anti-mouse CD45RB mAb on varying days, while groups II and IV receive no further antibody treatment. The effectiveness of the antibody treatment in preventing rejection of the skin graft is determined by comparing the length of time the black skin graft survives on the white recipient mice in the four groups.

Localized Graft-Versus-Host (GvH) Reaction

In vivo efficacy of the compounds is proven in a suitable animal model, as described, *e.g.*, in Ford et al, TRANSPLANTATION 10 (1970) 258. Spleen cells (1×10^7) from 6 week old female Wistar/Furth (WF) rats are injected subcutaneously an day 0 into the left hind-paw of female (F344 x WF)F_1 rats weighing about 100g. Animals are treated for 4 consecutive days and the popliteal lymph nodes are removed and weighed on day 7. The difference in weight between the two lymph nodes is taken as the parameter for evaluating the reaction.

Kidney Allograft Reaction in Rat

One kidney from a female Fisher 344 rat is transplanted onto the renal vessel of a unilaterally (left side) nephrectomized WF recipient rat using an end-to-end anastomosis. Ureteric anastomosis is also end-to-end. Treatment commences on the day of transplantation and is continued for 14 days. A contralateral nephrectomy is done seven days after transplantation, leaving the recipient relying on the performance of the donor kidney. Survival of the animal is taken as the parameter for a functional graft.

Freund's Adjuvant Arthritis

Efficacy against experimentally induced arthritis is shown using the procedure described, *e.g.*, in Winter & Nuss, ARTHRITIS & RHEUMATISM 9 (1966) 394; Billingham Davies, HANDBOOK OF EXPERIMENTAL PHARMACOL (Vane & Ferreira Eds, Springer-Verlag, Berlin) 50/II (1979) 108-144. OFA and Wistar rats (male or female, 150g body weight) are injected, *i.e.*, at the base of the tail or in the hind paw, with 0.1 ml of mineral oil containing 0.6 mg of lyophilized heat-killed Mycobacterium smegmatis. In the developing arthritis model, treatment is started immediately after the injection of the adjuvant (days 1-18); in the established arthritis model treatment is started on day 14, when the secondary inflammation is well developed (days 14-20). At the end of the experiment, the swelling of the joints is measured by means of a micro-caliper. ED_{50} is the oral dose in mg/kg which reduces the swelling (primary or secondary) to half of that of the controls.

In vivo treatment of NZB mice to inhibit onset of lupus-like autoimmune disease.

Mice of the New Zealand black-strain (NZB) die with widespread and diverse symptoms of hemolytic anemia, glomerulonephritis, and vasculitis, all very reminiscent of human systemic lupus erythematosus (SLE). The effectiveness of the invention compounds in treating SLE in humans is evaluated by treating newborn NZB mice with rat anti-mouse CD45RB mAb MB23G2 at varying times after birth and then analyzing treated and untreated mice for the onset of autoimmune disease, particularly for glomerulonephritis, which is also a prominent feature of human SLE.

Appropriate dosages of the Novel Compounds will of course vary, *e.g.*, depending on the condition to be treated (for example the disease type or the nature of resistance), the effect desired, and the mode of administration.

In general however satisfactory results are obtained on administration parenterally, *e.g.*, intravenously, for example by iv drip or infusion, at dosages on the order of from 0.01 to 2.5 up to 5 mg/kg, *e.g.*, on the order of from 0.05 or 0.1 up to 1.0 mg/kg. Suitable dosages for patients are thus on the order of from 0.5 to 125 up to 250 mg iv, *e.g.*, on the order of from 2.5 to 50 mg i.v. The Compounds may be administered daily or every other day or less frequently at diminishing dosages to maintain a minimum level of Compound in the blood during the antigen challenge, *e.g.*, following organ transplant or during the acute phase of an autoimmune disease.

Pharmaceutical compositions of the invention may be manufactured in conventional manner. A composition according to the invention is preferably provided in lyophilized form. For immediate administration it is dissolved in a suitable aqueous carrier, for example sterile water for injection or sterile buffered physiological saline. If it is considered desirable to make up a solution of larger volume for administration by infusion rather as a bolus injection, it is advantageous to incorporate human serum albumin or the patient's own heparinized blood into the saline at the time of formulation. The presence of an excess of such physiologically inert protein prevents loss of monoclonal antibody or fusion protein by adsorption onto the walls of the container and tubing used with the infusion solution. If albumin is used, a suitable concentration is from 0.5 to 4.50% by weight of the saline solution.

In clinical tests, for example, patients about to undergo kidney, liver or heart transplantation are selected for prophylactic therapy. On the day of transplantation, 2 hours before surgery, a first intravenous infusion of the chimeric CD45RB antibody of Example 2 is administered at a dose of 0.2 mg of each antibody per kg of body weight. Two days after surgery an identical infusion of the antibodies at 0.4 mg/kg of body weight is administered and then repeated at weekly intervals for one month. The intravenous infusions are prepared as

follows: the lyophilized antibodies are mixed together and dispersed into 100 ml sterile buffered saline containing 4.51% by weight of human albumin. This saline dispersion is administered to the patients over a 30 minute period.

The Compounds are also useful as diagnostic aids, as diagnostic reagents or as components of a diagnostic kit to identify particular sub-populations of leukocytes. The Compounds may be labeled, *e.g.*, fluorolabelled or radiolabelled, using conventional techniques. For example, 25 micrograms of monoclonal antibody in 0.25 ml of 0.12 M sodium phosphate, pH 6.8 is iodinated using 2 mCi ^{125}I and 10 micrograms chloramine T. After 5 minutes at 23°C, the reaction is stopped by the addition of 20 micrograms of sodium metabisulfite, 3 mg of KI and 1 mg of BSA. Iodinated protein is separated chromatographically. The labeled Compounds are exposed to a frozen tissue section, *e.g.*, from a patient exhibiting symptoms of graft rejection or acute autoimmune disease, exhibiting infiltration of leukocytes. Excess Compound is washed away, and bound Compound is assayed. Substantial binding of the Compounds to leukocytes present in the tissue section suggests that the majority of leukocytes involved are naive rather than memory leukocytes, thereby indicating that therapy with the Compounds and/or with immunosuppressants acting primarily on the T-cell mediated immune response, *e.g.*, Cyclosporin or FK-506, is appropriate.

Finally, the Compounds are useful in a screening assay to identify drugs capable of modulating the biological activity of CD45RB.

Thus, it is apparent that there has been provided, in accordance with the present invention, methods and products which will substantially benefit those with autoimmune diseases and those receiving organ transplants. While the invention has been described in conjunction with specific embodiments thereof, it is evident that many modifications and variations will be apparent to those skilled in the art.

For instance, it is now known that the epitope identified by the CD45RB mAb MB23G2 is encoded by the B exon of the leukocyte common antigen gene. Isolation of the epitope and compounds binding to the epitope are contemplated by this invention.

Accordingly, it is intended to include all such alternatives, modifications and variations set forth within the spirit and scope of the appended claims.

What is claimed is:

1. A method for treating or preventing a cell, tissue or organ transplant rejection in a recipient, comprising administering a pharmacologically effective dose of at least one compound which binds to the CD45RB leukocyte antigen, so as to inhibit a T-cell mediated immune response in the recipient to said transplant.

2. The method of claim 1, wherein said cell, tissue or organ transplant is allogeneic to recipient.

3. The method of claim 1, wherein said cell, tissue or organ transplant is xenogeneic to recipient.

4. The method of claim 1 wherein the compound binds to a neuraminidase sensitive epitope on said antigen.

5. The method of claim 1 wherein said binding inhibits leukocyte signal transduction by increasing tyrosine phosphorylation of phospholipase C-γ1.

6. The method of claims 1, 2, 3, 4 or 5 wherein said compound is selected from the group consisting of an anti-CD45RB monoclonal antibody, an antigen binding fragment thereof, or a mixture thereof.

7. The method of claim 6 wherein said compound is an anti-CD45RB monoclonal antibody.

8. The method of claim 6 wherein the compound does not bind to the CD45RO leukocyte antigen.

9. The method of claim 8 wherein said anti-CD45RB monoclonal antibody is a chimeric antibody comprising at least one of the complementary determining regions of MT3 or 6G3.

10. The method of claim 9 wherein said chimeric antibody also comprises at least one constant region from a human immunoglobulin molecule.

11. The method of claim 9 wherein said anti-CD45RB monoclonal antibody is MT3, 6G3 or MB23G2.

12. A method for increasing tyrosine phosphorylation of phospholipase C-γ1 in a mammalian cell, so as to treat a condition characterized by pathological proliferation of leukocytes, comprising: administering to the mammal a therapeutically effective amount of a compound which specifically binds to neuraminidase sensitive epitopes on leukocytes.

13. A method for preventing allograft or xenograft rejection in a transplant recipient, comprising: administering a therapeutically effective amount of a compound which specifically binds to neuraminidase sensitive epitopes of CD45RB of leukocytes.

14. The method of claims 12 or 13 wherein the leukocytes are T lymphocytes.

15. A method for preventing rejection of transplanted cells, tissues or organs in a mammalian transplant recipient comprising administering to the recipient a therapeutically effective amount of an anti-CD45RB monoclonal antibody or a therapeutic fragment thereof.

16. The method of claims 12, 13 or 15, further comprising administering at least one anti-inflammatory or immunosuppressive drug.

17. The method of claim 16, wherein said anti-inflammatory or immunosupressive drug is selected from the group consisting of cyclosporin, FK-506, Rapamycin, corticosteriods, mycophenolate mofetil, Leflunomide Anti-Lymphocyte Globulin, Deoxyspergualin and OKT-3.

18. A method for treating an autoimmune disease, comprising administering a pharmacologically effective dose, to a mammal having an autoimmune disease, of at least one compound which binds to the CD45RB leukocyte antigen, so as to inhibit a T cell mediated immune response.

19. The method of claim 18 wherein the compound is an anti-CD45RB monoclonal antibody, an antigen binding fragment thereof or a mixture thereof.

20. The method of claim 19 wherein the compound is an anti-CD45RB monoclonal antibody.

21. The method of claim 20 wherein the antibody is MT3, 6G3, or MB23G2.

22. The method of claim 20 wherein the antibody does not bind to the CD45RO leukocyte antigen.

23. The method of claim 18 wherein the autoimmune disease is multiple sclerosis, systemic lupus erythematosus or rheumatoid arthritis.

24. A chimeric monoclonal antibody to CD45RB of the IgG class, in which the variable domain is murine origin and a constant domain is human origin and which increases tyrosine phosphorylation of phospholipase C-γ1.

25. A chimeric monoclonal antibody to CD45RB of the IgG class, having a variable domain of murine origin and a constant domain of human origin, and which binds to a neuraminidase-sensitive epitope of CD45RB.

26. A pharmaceutical composition comprising an effective immunosuppressive amount of a ligand having a binding site for CD45RB, said ligand inhibiting leukocyte signal transduction by increasing tyrosine phosphorylation of phospholipase C-γ1, in combination with a pharmaceutically acceptable vehicle.

27. A pharmaceutical composition comprising an effective immunosuppressive amount of a ligand having an antibody binding domain to a neuraminidase sensitive epitope of CD45RB, in combination with a pharmaceutically acceptable vehicle.

28. The composition of claims 26 or 27 wherein the ligand is an antibody, an antibody fragment, an antibody derivative or an antibody mutant.

29. The composition of claim 28, wherein said antibody is 6G3, MT3 or MB23G2.

30. The composition of claim 26 or 27 wherein the amount of ligand inhibits the expression of at least one inflammatory cytokine.

31. The composition of claim 30 wherein said cytokine is γ-interferon, tumor necrosis factor α or intercellular adhesion molecule-1.

FIG. 1

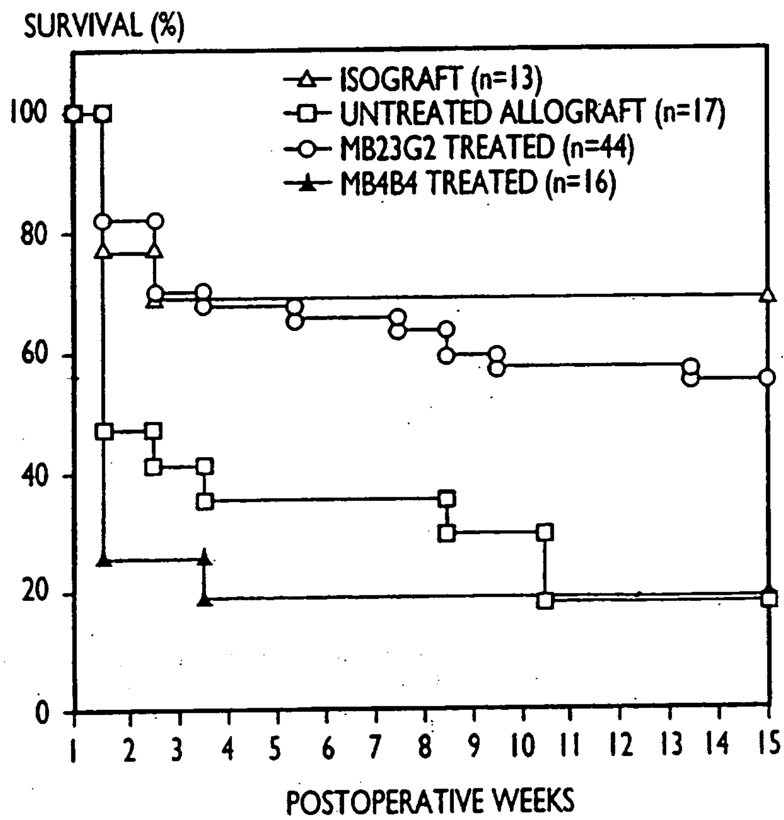

SURVIVAL (%)
ISOGRAFT (n=13)
UNTREATED ALLOGRAFT (n=17)
MB23G2 TREATED (n=44)
MB4B4 TREATED (n=16)
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
POSTOPERATIVE WEEKS

FIG. 2

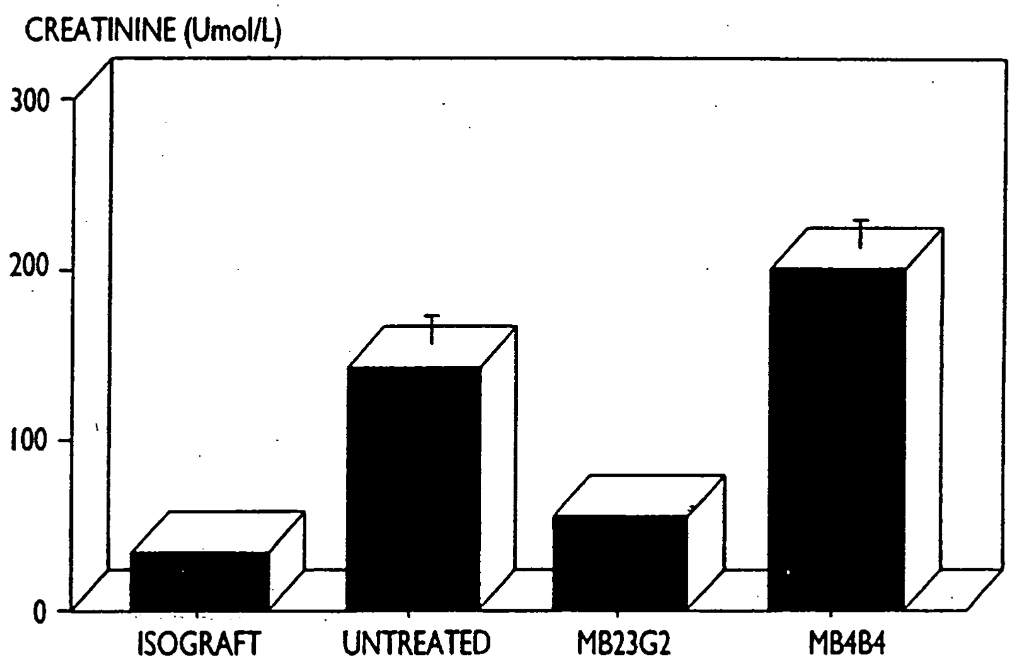

CREATININE (Umol/L)
300
200
100
0
ISOGRAFT
UNTREATED
MB23G2
MB4B4

FIG. 3A

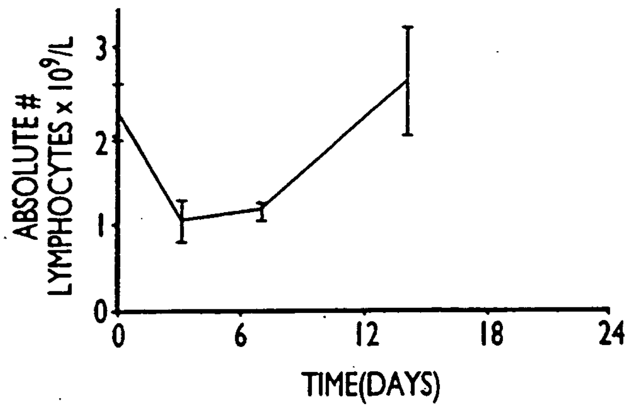


FIG. 3B

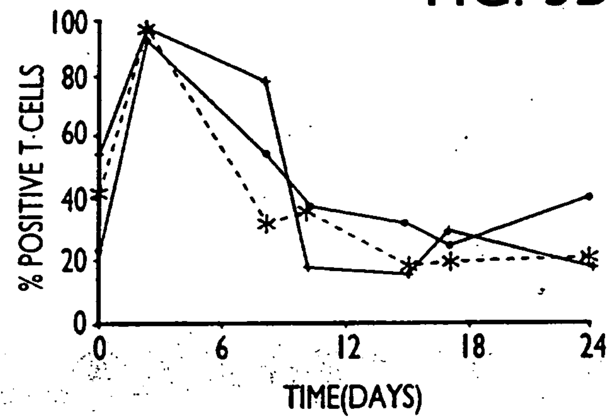


FIG. 3C

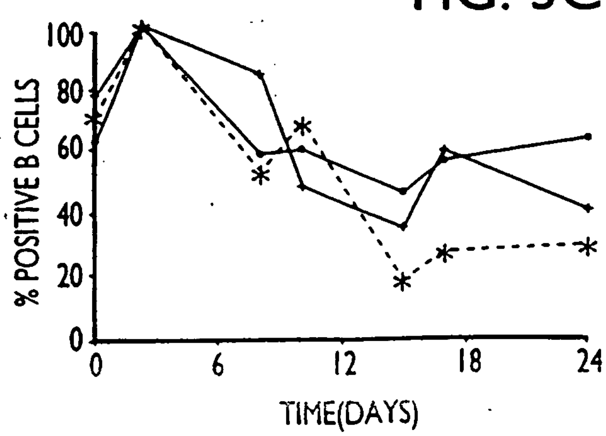
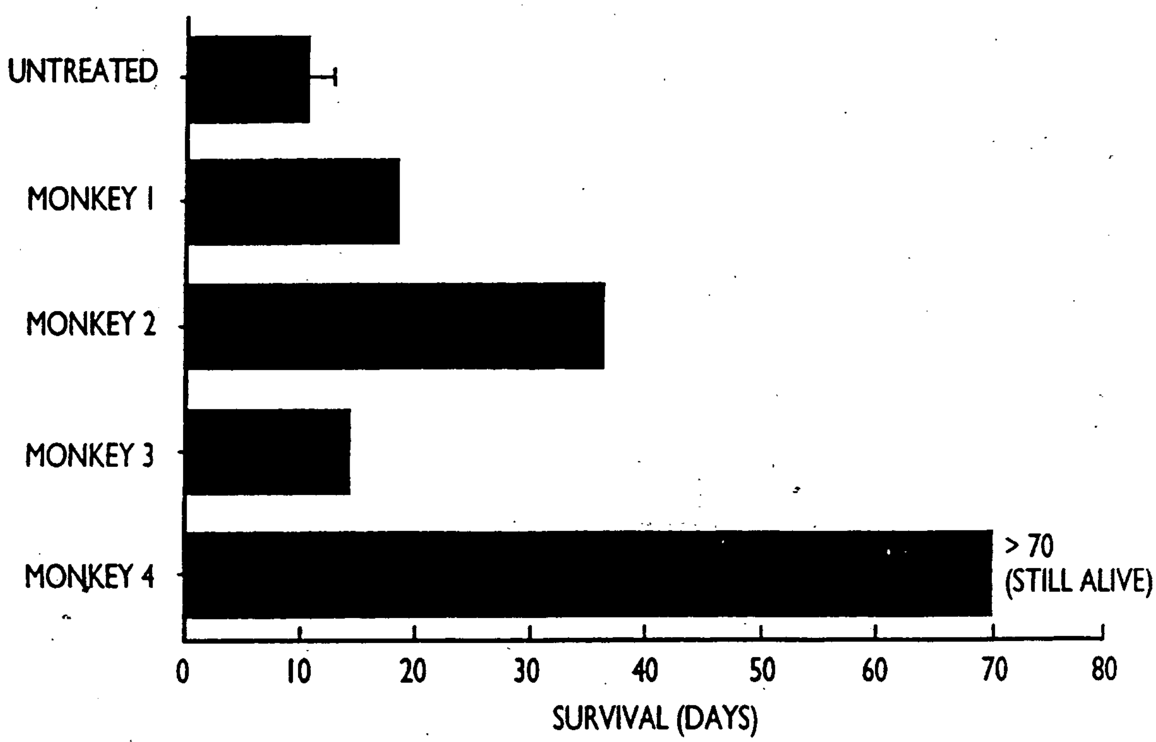


FIG. 4


UNTREATED
MONKEY I
MONKEY 2
MONKEY 3
MONKEY 4
> 70
(STILL ALIVE)
0
10
20
30
40
50
60
70
80
SURVIVAL (DAYS)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/05204

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 C07K16/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TRANSPLANTATION PROCEEDINGS, vol. 27, no. 1, February 1995, page 389 XP000590838 ZHANG Z. ET AL: "Prevention and reversal of renal allograft rejection by monoclonal antibody to CD45RB in the mouse model" see the whole document ---	1-31
X	TRANPLANTATION PROCEEDINGS, vol. 27, no. 1, February 1995, page 398 XP000590837 OGASA N. ET AL: "Altered expression of cytokine genes by CD45RB monoclonal antibody in renal allograft rejection" see the whole document ---	1-31

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
25 July 1996	01.08.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Fernandez y Branas, F

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TRANSPLANTATION, vol. 54, no. 4, 1992, pages 724-729, XP000575872 LAZAROVITS A.I. ET AL: "Inhibition of alloreactivity in vitro by monoclonal antibodies directed against restricted isoforms of the leukocyte-common antigen (CD45)" cited in the application see the whole document	1-31
A	JOURNAL OF MOLECULAR AND CELLULAR IMMUNOLOGY, vol. 4, 1988, pages 71-85, XP000590834 BIRKELAND M.L. ET AL: "Epitopes on CD45R [T200] molecules define differentiationantigens on murine B and T lymphocytes" see page 80 - page 81	1-31
A	JOURNAL OF IMMUNOLOGY, vol. 147, no. 1, 1991, BALTIMORE US, pages 218-223, XP000575807 POPPEMA S. ET AL: "Antibody MT3 is reactive with a novel exon B-associated 190-kDa sialic acid dependent epitope of the leukocyte common antigen complex" *page 218, column 2, "materials and methods"*	1-31
A	LABORATORY INVESTIGATION, vol. 64, no. 6, 1991, pages 844-854, XP002009416 RAYMOND LAI ET AL: "Tissue distribution of restricted leukocyte common antigens" see table 2	1-31
A	EP,A,0 449 769 (SANDOZ LTD ;ROYAL FREE HOSPITAL SCHOOL OF (GB)) 2 October 1991 see page 3 - page 4	1-31
A	IVAN M. ROITT: "ENCYCLOPEDIA OF IMMUNOLOGY" 1992 , ACADEMIC PRESS , LONDON XP002009418 see page 142 - page 144	18
T	NATURE, vol. 380, 25 April 1996, LONDON GB, pages 717-720, XP002009417 LAZAROVITS A. ET AL: "Prevention and reversal of renal allograft rejection by antibody against CD45RB" see the whole document	1-31

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 96/05204

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-23
because they relate to subject matter not required to be searched by this Authority, namely:
Although these claims are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

International Application No
PCT/US 96/05204

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0449769	02-10-91	AU-B-	635401	18-03-93
		AU-B-	7290991	19-09-91
		CA-A-	2038279	17-09-91
		DE-D-	69100768	27-01-94
		DE-T-	69100768	11-05-94
		ES-T-	2061216	01-12-94
		JP-A-	4316600	06-11-92